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Flexible metabolic pathway construction using modular and divisible selection gene regulators

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Abstract

Genetic selections are important to biological engineering. Although selectable traits are limited, currently each trait only permits simultaneous introduction of a single DNA fragment. Complex pathway and strain construction however depends on rapid, combinatorial introduction of many genes that encode putative pathway candidates and homologs. To triple the utility of existing selection genes, we have developed divisible selection in *Saccharomyces cerevisiae*. Here, independent DNA fragments can be introduced and selected for simultaneously using a set of split hybrid transcription factors composed of parts from *Escherichia coli* LexA and *Herpes simplex* VP16 to regulate one single selectable phenotype of choice. Only when co-expressed, these split hybrid transcription factors promote transcription of a selection gene, causing tight selection of transformants containing all desired DNA fragments. Upon transformation, 94 percent of the selected colonies resulted strictly from transforming all three modules based on ARS/CEN plasmids. Similarly when used for chromosome integration, 95 percent of the transformants contained all three modules. The divisible selection system acts dominantly and thus expands selection gene utility from one to three without any genomic pre-modifications of the strain. We demonstrate the approach by introducing the fungal rubrofusarin polyketide pathway at a gene load of 11 kb distributed on three different plasmids, using a single selection trait and one yeast transformation step. By tripling the utility of existing selection genes, the employment of divisible selection improves flexibility and freedom in the strain engineering process.

Keywords: Pathway construction, selection system, chromosomal integration, synthetic biology, split transcription factor

Abbreviations: AD: Activation domain, DBD: DNA-binding domain, TF: Transcription factor, DS: Divisible selection

1 Introduction

Metabolic engineering and synthetic biology research necessitates frequent introduction of several pieces of heterologous DNA to host strains at ever-increasing pace (Bornscheuer et al., 2012; Boyle and Silver, 2012; Keasling, 2012; Tyo et al., 2007). The rising complexity of reconstructed metabolic pathways demands high flexibility and freedom in these genetic manipulations to characterize the full space of variables.

Various approaches have been developed for combinatorial assembly of single or several pathway steps using yeast homologous recombination, software-guided cloning or simply large random clone assemblies (Genee et al., 2014; Gibson et al., 2010; Naesby et al., 2009; Shao et al., 2009). Systematic sampling of such biological variables as expression strength, codon usage, truncations and homologs is a strategy for developing metabolic pathways, where optimally performing strains may result from particular combinatorial clones. Freedom and flexibility in gene introduction methods are thus of importance.

Irrespective of gene introduction strategy, the selection genes that aid transformation with foreign DNA still follow the same principle of "one selectable trait, one selection gene". Consequently, selection genes are iteratively removed to allow recycled use when multi-gene systems are constructed, since selective traits are limited. Indeed, many strain construction methods are based on different approaches to recycling, using loop-out mechanisms based on recombinases or endogenous homologous recombination in increasingly streamlined implementations (Da Silva and

Srikrishnan, 2012; Hegemann and Heick, 2011; Jensen et al., 2013; Mikkelsen et al., 2012; Siddiqui et al., 2014; Wingler and Cornish, 2011). Still, it would increase speed if more independent gene introductions were possible using the limited number of available selectable traits. Indeed, new selection genes are routinely identified to further expand strain construction freedom (Regenberg and Hansen, 2000; Solis-Escalante et al., 2013). Whilst some are dominant in action, the recessive nature of most auxotrophic selection genes requires pre-modification of the receiving host strain by deletion of complementing prototrophic genes. The converse dominant selection genes typically involve antibiotics for maintenance of selection pressure, including harmful, costly agents such as bleomycin and hygromycin. Present-day pathway construction can thus yield strains requiring a mixture of selection pressures (Nielsen et al., 2014).

Saccharomyces cerevisiae is an important host for production of biofuels and commodity chemicals as well as structurally more advanced secondary metabolites, and the pathway complexities can only be expected to rise (Nielsen et al., 2013; Siddiqui et al., 2011).

As a modular and conceptually novel approach to multiply the utility of each selectable trait in *S. cerevisiae*, we here design and develop divisible selection based on split transcription factors (TFs). With divisible selection, three independent DNA fragments can be introduced simultaneously at the load of a single selection phenotype of choice. This is possible by dividing the regulation of selectable phenotypes into modules composed of hybrid split TFs. Exclusively when co-expressed, these reconstitute activation of a tight selection gene promoter, yielding

efficient selection for all associated DNA fragments. The split TFs of divisible selection are designed to function dominantly and therefore expand the utility of an already functional selection gene from one to three, and possibly more, DNA fragments without any strain pre-modifications. Split divisible phenotypes could therefore take many applications within biological engineering. As a proof-of-principle for the system, we here employ divisible selection within metabolic engineering to reconstruct the *Fusarium graminearum* three-step polyketide pathway to rubrofusarin in *S. cerevisiae* by expression of the biosynthetic genes from three individual plasmids under one selectable trait.

2 Methods and materials

2.1 Strains

All characterization of the system efficiencies was performed in *Saccharomyces cerevisiae* CEN.PK2-1C (MATa; *ura3-52; trp1-289; leu2-3,112; his3Δ 1; MAL2-8^{C}; SUC2*). The three-step rubrofusarin polyketide pathway was introduced into *S. cerevisiae* CEN.PK2-1C pre-transformed with pRS413-npgA (CEN.PK2-1C-npgA) yielding the strains listed in Table 1.

All molecular cloning was performed using transformation into *Escherichia coli* XL1 chemically competent cells (Stratagene).

Table 1 Strains analyzed for ability to maintain plasmids for rubrofusarin production given the indicated method of selection based either on classical selection genes (cl) or divisible selection (ds) using a hybrid DNA-binding domain (DBD) and activation domain (AD). All strains were generated from the CEN.PK2-1C-npgA parent strain.

Strain	Pathway plasmids	Selective gene
cl-rub	pRS416-PKS12	*URA3*
	pRS414-aurZ	*TRP1*
	pRS415-aurJ	*LEU2*
ds-rub	pDS1-PKS12	*ds1-URA3*
	pDS2-aurZ	*ds2-DBD*
	pDS3-aurJ	*ds3-AD*

2.2 Materials

Unless otherwise stated, reagents were purchased from Sigma-Aldrich. Synthetic complete (SC) medium was prepared with 1.4 g/L synthetic complete drop-out mix lacking uracil, tryptophan, leucine and histidine (Y2001), 6.7 g/L yeast nitrogen base without amino acids (Y0626) and 20 g/L D-glucose, pH standardized to 5.6. Cu^{2+} for gene induction was added from a stock solution of 100 mM $CuSO_4$. When SC was supplemented with additional amino acids, 60 mg/L leucine, 20 mg/L uracil, 20 mg/L histidine-HCl and 20 mg/L tryptophan was added. Oligonucleotides were purchased from Integrated DNA Technologies.

2.3 Construction of plasmids

Plasmids were assembled by uracil-excision (USER) cloning of purified PCR fragments, except for pDS2-aurZ, which was cloned by Gibson assembly of a purified PCR fragment into a pDS2 vector linearized with SmaI. Gibson assembly was performed using 2x Gibson assembly master mix (New England Biolabs) according to

the manufacturer's protocol. The general method for USER cloning was based on agarose gel-purification of the PCR products amplified with DNA polymerase X7 (Nørholm, 2010). These were mixed and incubated in an equimolar 20 μL reaction with 0.5 μL USER enzyme (New England Biolabs) and 0.5 μL DpnI FastDigest (Thermo Scientific) in FastDigest buffer at 37 degrees C for 1-2 hours. Following 25 subsequent minutes at room temperature, 2.5 μL reaction was transformed into chemically competent *E. coli*. Correctly cloned plasmids were identified using restriction analysis and DNA sequencing. The detailed use of oligonucleotides for assembly of all plasmids and origin of parts are listed in supplementary material.

2.4 Plasmids

All plasmids developed and characterized for divisible selection are listed in Table 2 and all plasmids for production of rubrofusarin pathway are listed in Table 3.

Table 2 Plasmids for use in divisible selection and conventionally selected plasmids (pRS41x) used for comparison. All vectors propagate in *S. cerevisiae* using the indicated method and in *E. coli* using pUC origin of replication and ampicillin resistance. NLS abbreviates nuclear localization signal. Unless otherwise stated, gene source is *S. cerevisiae.*

Plasmid name	**Selection gene promoter**	**Selection gene ORF**	**Selection gene terminator**	**Propagation in yeast by**	**Reference**
pDS1U	$8op_{lexA}$-*SPO13*	*URA3*	*K. lactis URA3*	CEN/ARS	This study
pDS1Um				2 micron	This study
pDS1U-X2				Chromosome integration	This study

pDS1H	$8op_{lexA}$-*SPO13*	*HIS3*	*K. lactis URA3*	CEN/ARS	This study
pDS2	*A. gossypii TEF1*	*NLS–E. coli lexA–krev1*	*A. gossypii TEF1*	CEN/ARS	This study
pDS2m				2 micron	This study
pDS2-X3				Chromosome integration	This study
pDS3	*A. gossypii TEF1*	*NLS–H. simplex VP16(AD)–ralGDS*	*A. gossypii TEF1*	ARS/CEN	This study
pDS3m				2 micron	This study
pDS3-X4				Chromosome integration	This study
pDS1.2U	$8op_{lexA}$-*SPO13*	*URA3*	*K. lactis URA3*	ARS/CEN	This study
pDS1.3U	$4op_{lexA}$-*SPO13*	*K. lactis URA3*	*K. lactis URA3*	ARS/CEN	This study
pRS414-empty	*TRP1*			ARS/CEN	(Sikorski and Hieter, 1989)
pRS415-empty	*LEU2*			ARS/CEN	(Sikorski and Hieter, 1989)
pRS416-empty	*URA3*			ARS/CEN	(Sikorski and Hieter, 1989)

Table 3 Divisible selection plasmids developed to reconstruct the *F. graminearum* (*Fg*) rubrofusarin polyketide pathway in *S. cerevisiae* CEN.PK2-1C-npgA based on uracil prototrophy and benchmark plasmids with the same pathway genes and classical auxotrophic selection genes. The respective pathway open reading frames (ORF) were expressed with the indicated promoter and terminators from *S. cerevisiae*. All plasmids propagate in *S. cerevisiae* based on a CEN/ARS origin.

Plasmid name	**Pathway cassette (promoter-ORF-terminator)**	**Selective gene**	**Reference**
pDS1U-	p_{CUP1}-*FgPKS12*-t_{ADH1}	*ds1-URA3*	This study

PKS12			
pDS2-aurZ	p_{TEF1}-*FgaurZ*-t_{ENO2}	*ds2-DBD*	This study
pDS3-aurJ	p_{GPD1}-*FgaurJ*-t_{CYC1}	*ds3-AD*	This study
pRS416-PKS12	p_{CUP1}-*FgPKS12*-t_{ADH1}	*URA3*	(Rugbjerg et al., 2013)
pRS414-aurZ	p_{TEF1}-*FgaurZ*-t_{ENO2}	*TRP1*	(Rugbjerg et al., 2013)
pRS415-aurJ	p_{GPD1}-*FgaurJ*-t_{CYC1}	*LEU2*	(Rugbjerg et al., 2013)
pRS413-npgA	p_{PYK1}-*npgA*-t_{TEF1}	*HIS3*	(Rugbjerg et al., 2013)

All plasmids constructed in this study will be deposited at the Addgene repository.

2.5 Transformation of *S. cerevisiae*

S. cerevisiae was transformed using lithium acetate-type transformation based on a high-efficiency protocol (Gietz and Schiestl, 2007) with a few minor adjustments: A single colony was pre-cultured overnight at 30 degrees C, 250 rpm shaking in 10 mL yeast peptone dextrose (YPD) medium. On the day of transformation, individual main cultures of 10 mL YPD were each inoculated to OD_{600} = 0.20 and cultured for 4 hours at 30 degrees C, 250 rpm shaking. The cell pellet of each was used per transformation. For chromosomal integration, to liberate 3 μg of each integration construct from its vector, 5 μg of each the integration vector were first linearized with SmiI FastDigest (Thermo Scientific) in a 40 μL reaction with 1x Fastdigest buffer for 2 hours at 37 degrees C and purified with a PCR clean-up kit (Macherey-Nagel) with

elution into 20 μL H_2O. The exact PEG_{3350} concentration was critical for good transformation efficiencies, and test titrations of every 50% PEG_{3350} batch were made to assure this by varying the volume of the batch +/- 10 percent in 2 percent point increments. The resultant optimum was used through compensation by adding less/more H_2O to the transformation mixture. Gentle final resuspension of the transformed cells in H_2O was also important for the transformation efficiency: After removal of the transformation mix supernatant and addition of H_2O, the sample was left to recover at room temperature for ten minutes prior to gentle resuspension using a pipette, first mildly tapping on the cell pellet before slowly pipetting it up and down two-three times. For chromosomal integrations this recovery was extended to twenty minutes. Each transformation was plated on one or two plates of synthetic complete (SC) medium lacking the appropriate selection nutrient. Colonies were counted following 65 hours of incubation at 30 degrees C. All transformations described were carried out in three or six replicates and no replicates were conducted in parallel.

2.6 Reconstruction of fungal polyketide pathway by divisible selection

S. cerevisiae CEN.PK2-1C-npgA was first constructed through transformation of *S. cerevisiae* CEN.PK2-1C with pRS413-npgA. Next, this strain was transformed with the plasmids pDS1U-PKS12, pDS2-aurZ and pDS3-aurJ (Table 2) in a single transformation step as described in section 2.5. After heat shock, the transformed cells were allowed to recover in YPD for 1 hour at room temperature prior to removal of YPD and plating. The exact influence of recovery based on YPD relative to H_2O was not evaluated. Selection of transformants was performed on SC -uracil plates.

Following incubation at 30 degrees C, individual transformants were re-plated to SC -uracil -histidine plates with 100 μM Cu^{2+} to induce the pathway and also select for maintenance of the previously transformed pRS413-npgA plasmid.

2.7 Extraction of *S. cerevisiae* DNA and PCR validation of presence of *ds* modules

A single colony was dissolved in 100 μL solution of 200 mM lithium-acetate, 1 % sodium dodecyl sulfate and incubated at 70 deg. C for 5 minutes. 300 μL 99 % ethanol was added and the cells were pelleted by centrifugation at 15,000 g, 3 minutes and washed in 400 μL 70 % ethanol. The cells were again pelleted and resuspended in 100 μL H_2O, after which the cells were pelleted at 15,000 g for 15 seconds. From the supernatant, 1 μL was used as template DNA for a PCR to validate presence of the three divisible selection plasmids using primers, which specifically anneal to unique parts of the ds modules: ds1: P55 + ID399 (expected size: 364 bp), ds2: P191 + P283 (expected size: 1239 bp) and ds3: P111 + P230 (expected size: 608 bp) (sequences in supplementary material Table S3). Site-specific integration by double-crossover on *S. cerevisiae* chromosome X was tested as devised in the Easyclone concept (Jensen et al., 2013) using the primer 5'-AGGTCGCUCATCGCACGC together with the following primers, which all anneal to loci immediately upstream of the three respective homologous recombination target areas: X-2: 5'-TGCGACAGAAGAAAGGGAAG (expected size: 700 bp), X-3: 5'-TGACGAATCGTTAGGCACAG (expected size: 887 bp), X-4: 5'-CTCACAAAGGGACGAATCCT (expected size: 810 bp).

And similarly for the downstream target areas using the primer 5'-ACCCAATTCGCCCTATAGTGAGTCG together with the chromosome-specific

primers X-2: 5’- GAGAACGAGAGGACCCAACAT (expected size: 1063 bp), X-3: 5’- CCGTGCAATACCAAAATCG (expected size: 757 bp), X-4: 5’- GACGGTACGTTGACCAGAG (expected size: 746 bp)

3 Results

3.1 Design of divisible selection

To develop effective divisible selection in *S. cerevisiae*, we identified five important system criteria. 1) Stringency: Selection should be highly efficient for all associated parts and disallow false selection at incomplete uptake of all parts. 2) Modularity: The ability to divide a selection phenotype into multiple co-dependent entities should be independent of the actual selection phenotype i.e. also function when plugging in a different selection gene ORF. 3) Dominance: Pre-adaptation of the host strain should not be necessary in order to use divisible selection instead of the corresponding single selection gene. 4) Stability: The system should use few endogenous parts from the host strain to minimize risks of recombination and crosstalk. 5) Scalability: The concept should be scalable towards wider division, such as split of TFs into additional components or simultaneous use of multiple, orthogonal TF networks.

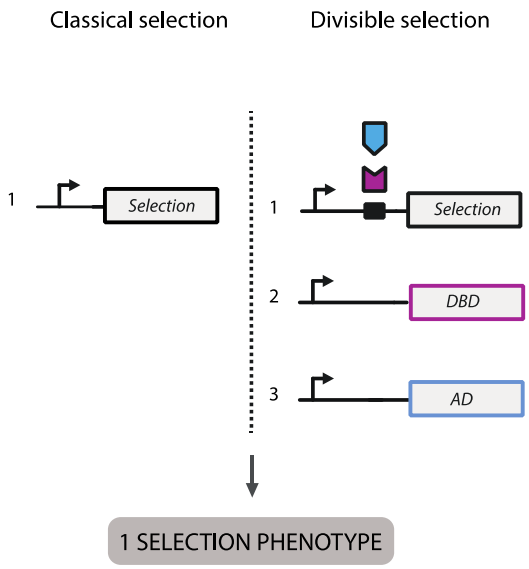

Figure 1 Design of divisible selection in *S. cerevisiae*. Unlike classical selection, divisible selection expands a single selectable trait to introduce multiple DNA fragments using split transcription factors. The selection gene is activated by the fully reconstituted transcription factor based on a hybrid DNA-binding domain (DBD) and hybrid transcriptional activation domain (AD).

To meet these criteria, we designed divisible selection as three individually expressed modules (Fig. 1) that we could link physically to three independent DNA fragments (plasmids). The modules consist of: DS1) A selection gene ORF driven by a promoter, tightly responsive to: DS2) A DNA-binding protein fused to a protein-interacting domain, cognate to: DS3) Another protein-interacting domain fused to a transcription activation domain. The TF-based approach would ensure modularity since other selection gene ORFs can be swapped in and out. To achieve high stringency, we engineered a synthetic promoter based on the *SPO13* promoter, which

features a mitotic UME6 repressor-binding site to suitably abolish leaky expression as previously utilized (Vidal et al., 1996). UME6 represses *SPO13* promoter activity in mitotic cells (Mitchell, 1994), thus rendering the TF response tighter. This is important since even very low expression of auxotrophic selection genes typically reconstitute prototrophy.

To develop divisible selection to act dominantly on existing selection phenotypes, we engineered the split TFs using proteins heterologous to *S. cerevisiae* to avoid interactions with endogenous repressors or promoters. Finally, we expressed the split TF modules from promoters not present in the *S. cerevisiae* genome to limit unintended recombination.

3.2 Detailed design composition

The divisible selection module *ds1-URA3* was engineered with the classical *URA3* gene under control of a 157 bp *SPO13* promoter fragment (Fig 2A). Upstream of the promoter fragment, we inserted four natural and synthetic DNA-binding sites specific for *Escherichia coli* LexA at various *in vitro*-based K_d values down to 0.8 10^{-9} M (Zhang et al., 2010) to achieve high saturation as soon as few LexA proteins enter the cell nucleus. As terminator, the *URA3* terminator from *Klyveromyces lactis* was used.

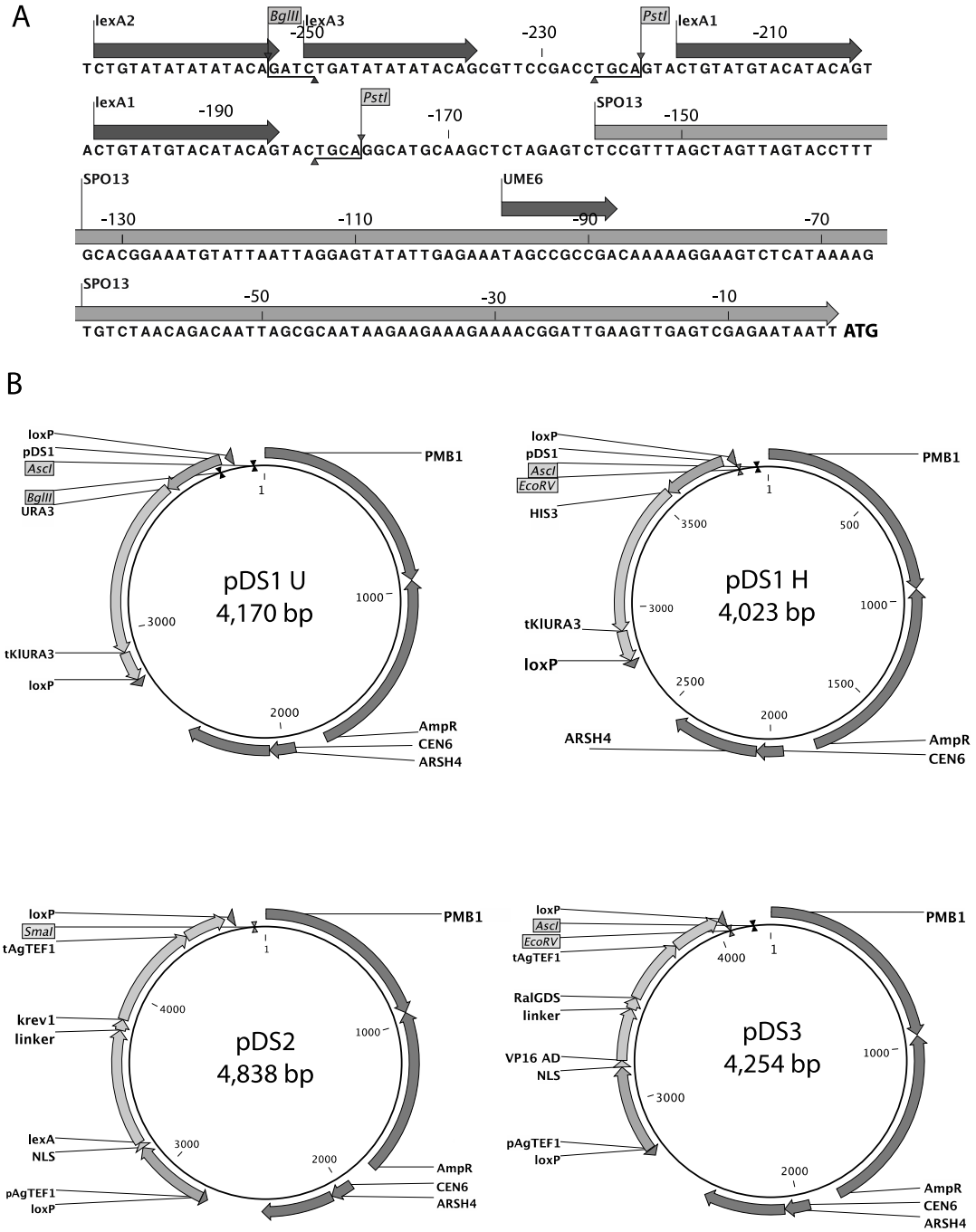

Figure 2. Sequence composition of core divisible selection modules and their vectors. A) DNA sequence of the hybrid DS1 promoter featuring elements of the *S. cerevisiae SPO13* promoter and upstream different LexA-binding sites. B) Plasmid maps showing the parts orchestration for the three main divisible selection modules and their propagating ARS/CEN vectors.

The *ds2-DBD* module consists of the *E. coli* LexA DNA-binding repressor in an N-terminal fusion to a nucleus localization sequence (NLS). Using an RSNQTSLYKKAGSAAAPFT linker, LexA is C-terminally fused to a Krev-1 (Rap1A) protein, known for its functional protein interaction to RalGDS (Herrmann et al., 1996). For expression of the hybrid protein, a heterologous *TEF1* promoter and terminator pair from *Ashbya gossipy* (*Ag*) was chosen.

The *ds3-AD* module was an N-terminal fusion of NLS to the potent activation domain of *Herpes simplex* VP16 linked C-terminally to RalGDS using an SNQTSLYKKAGSAAAPFT linker. Expression was driven from the *AgTEF1* promoter and terminator.

Each module was inserted in a CEN/ARS-propagated vector derived of pRS416, but free of yeast selection genes (Fig. 2B). loxP sites flank all modules to add optional compatibility to recycling systems using cre recombinase-based recycling of selection genes.

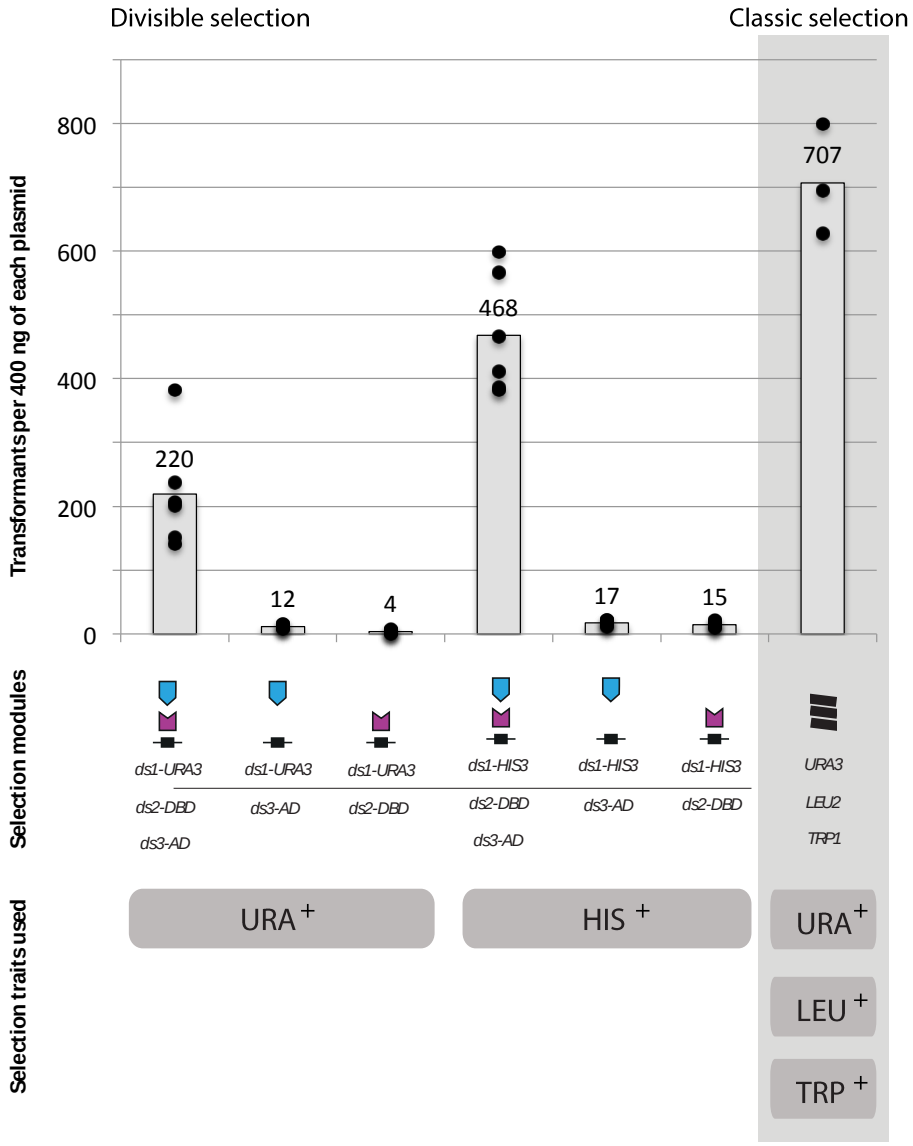

Figure 3 Tight selection of three plasmids under one selectable trait by divisible selection. Transformation efficiencies (average CFU per 400 ng of each plasmid) in *S. cerevisiae* CEN.PK2-1C transformed with incomplete or complete divisible selection modules (*ds1-HIS3/URA3* , *ds2-DBD* *and ds3-AD*) under selection on SC –ura or SC –his, or SC –ura, -trp, -leu (with control classic selection genes). CFUs were counted following 65 hours at 30 deg. C. Black points show values from individual replicate transformations (n = 6, except for classical plasmids where n = 3).

3.3 Selection for three different plasmids activating a single selection phenotype

To characterize the performance of divisible selection, all three modules *ds1, ds2* and *ds3* were simultaneously transformed into the *ura3*-deficient *S. cerevisiae* CEN.PK2-1C, and an average of 220 transformants per 400 ng of each plasmid formed colonies after 3 days incubation on SC -ura (Fig. 3). Theoretically, false activation of the *ds1* module in absence of *ds2* (DBD) and/or *ds3* (AD) has potential to cause false-positive selection. Thus, the vectors containing *ds1* and *ds3* were used as negative transformation control to specifically characterize possible transcriptional activity from the hybrid DBD protein and selection gene ORF. Following 3 days, an average of 4 colonies were observed after transformation with *ds1-URA3* and *ds2*, whereas 12 colonies were observed with *ds1-URA3* and *ds3*, indicating that the selection system was tight with a 94 % occurrence of true-positive transformants (Fig. 3). The few false-positive colonies were generally smaller than the true-positive colonies. The efficiency was further compared to transformation of three plasmids (pRS414, pRS415 and pRS416) selected for using the classical auxotrophic selection genes *LEU2, TRP1* and *URA3* to benchmark the general co-transformation efficiency. These plasmids resulted in an average 3-fold higher colony count at 707 transformants per 400 ng of each three plasmids. The reason for this advance in efficiency was not apparent, but we suspected suboptimal transcriptional activation of *ds1* to be responsible. To modify this however, it would be important to not simultaneously increase the frequency of false-positive transformants and we concluded the resulted transformation efficiency to be satisfactory for most applications.

A few preliminary constructs of the *ds1* module were also evaluated in slightly modified pRS41x-type vectors, testing fewer lexA binding sites and a heterologous *K. lactis URA3* sequence instead of the *S. cerevisiae URA3*. Remarkably, while no difference in colony formation was seen at 3 days, a considerate number of false-positive transformants formed colonies when using fewer binding sites and the *K. lactis URA3* following prolonged incubation at 30 degrees C for 6 days (supplementary material).

3.4 Modular exchange of selection phenotype from Ura$^+$ to His$^+$

To test the modularity of divisible selection and its ability to expand other selectable traits, we exchanged the *URA3* ORF in *ds1* with the ORF of *HIS3* from *S. cerevisiae*. Transformation with the three modules *ds1-HIS3, ds2* and *ds3* resulted in an average of 468 transformants after 3 days incubation. 17 transformants resulted from the control transformation with only *ds1-HIS3* and *ds3*, relative to 15 colony-forming cells with *ds1-HIS* and *ds2*, indicating that the system robustly could shift to a different prototrophy (Fig. 3). Thus, the simple exchange of the selection phenotype demonstrates the versatility of the concept, and indicates that more selectable traits could be modularly divided.

3.5 Divisible selection with copy number-fluctuating plasmids (2-micron)

Elevation of gene copy number can lead to increased formation of the metabolic products. To this end, the multi-copy 2-micron yeast plasmid is sometimes utilized, although its copy number varies considerably per cell (Da Silva and Srikrishnan, 2012; Jensen et al., 2013). To test how this plasmid type functions with the divisible selection system, we cloned *ds1U*, *ds2* and *ds3* into three plasmids with 2-micron origins. The plasmids were transformed simultaneously, while omitting the *ds2* plasmid as a control for false-positive transformants.

Following three days of incubation, an average of 327 transformants (std. dev. = 85) were visible, relative to the false-positive control omitting the *ds2* module, which reached 30 visible transformants (std. dev. = 4). However, on day four more transformants appeared on especially the false-positive control plates, and on day five this number reached a proportion of around 50 % the number of true-positive transformants. This high occurrence of false-positive transformants could be a consequence of selecting for amplification of the *ds1U* plasmid copy number, allowed by the 2-micron origin over time. Such an effect is similar to plasmid amplification methods that utilize weakly expressed selection genes (Chen et al., 2012; Erhart and Hollenberg, 1983): Elevated *URA3* baseline expression may eventually provide enough *URA3* to reconstitute the Ura^+ phenotype in absence of *ds2* or *ds3*. Accordingly, the copy-number fluctuation of the 2-micron plasmid renders it a suboptimal plasmid system for use with the divisible selection system compared to the centromeric plasmids.

3.6 Three simultaneous chromosomal integrations selected under one selection phenotype

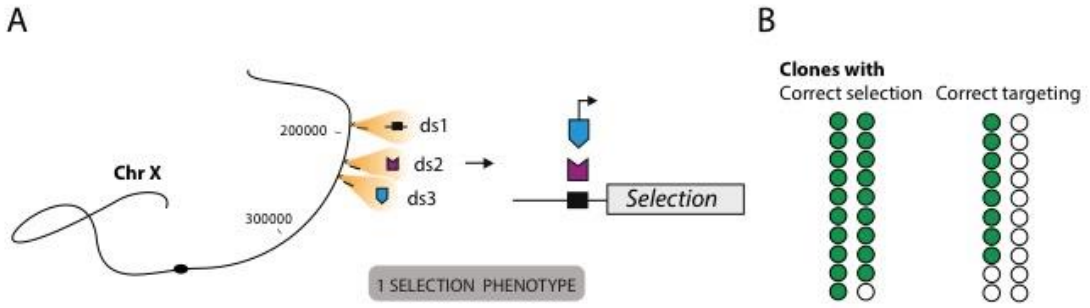

Figure 4 Chromosomal integration of three DNA fragments at different sites activating a single selection phenotype. A) Simultaneous gene insertion at three chromosome X loci in *S. cerevisiae* reconstituting a split hybrid transcription factor to activate a single selection phenotype. Genomic positions are correct relative to the two base-pair marks shown, while the remaining chromosome is overdrawn, B) PCR validation subsequently resulted in 95 % clones with all three *ds* modules present (correct selection), and 40 % of the clones showed targeting at the intended chromosomal location.

Chromosomal integration of genes is frequently used in metabolic engineering due to the copy number stability offered, e.g. alleviating selection for maintenance. Thus, to test whether the utility of a selection phenotype also could be tripled for chromosomal integration, we cloned the three ds modules *ds1U*, *ds2* and *ds3* into three integration vectors from the Easyclone concept (Jensen et al., 2013). The Easyclone vectors target three distinct loci on *S. cerevisiae* chromosome X (Mikkelsen et al., 2012) through homologous recombination of 0.5 kb upstream and downstream flanking sequences (Fig. 4A). Such simultaneous integration at three distinct loci is rarely reported, which may also be due to the fact that a single cell should both take up three different DNA fragments and integrate each of them correctly. Since high

transformation efficiency would be required to obtain three simultaneous integrations, a generous amount of DNA (3 μg of each *ds* module) was applied during transformation. Following three days' incubation, 20 transformants were isolated on SC plates lacking uracil. First, we assayed the selection stringency of the resulting clones. PCR on gDNA extracted from the transformants revealed presence of *ds1* and *ds2* in all twenty colonies, whereas *ds3* could be confirmed in nineteen, leading to an overall estimated efficiency of 95 % (Fig. 4B). Further, the single transformant lacking *ds3* appeared to grow much slower when re-streaked to selective medium. The efficiency of chromosomal targeting in yeast not only depends on the performance of the selection mechanism, but may also depend on the specific integration constructs and target loci. Next, we tested this targeting efficiency by PCR using locus-specific primers. In 40 % of our transformants, site-specific insertion of all three *ds* modules could be verified by PCR. Simultaneous gene targeting at three loci has previously been shown with 44 % targeting efficiency using classical auxotrophic selection genes (Jensen et al., 2013), which thus agreed very well.

3.7 Proof-of-principle construction of fungal polyketide pathway

To prove the divisible selection principle for introduction of a multi-gene metabolic pathway, we chose a fungal secondary metabolic pathway as test case. Into each of the three pDS vectors, we inserted one of the three genes known to reconstruct the pathway leading from endogenous acetyl CoA and malonyl CoA to rubrofusarin *PKS12*, *aurZ* and *aurJ* (Rugbjerg et al., 2013). These genes encode respectively an iterative type I polyketide synthase, dehydrogenase and O-methyltransferase (Fig.

5A). Since the host yeast strain harbored a gene encoding a general polyketide synthase-activating phosphopantetheinyl transferase NpgA expressed from a fourth CEN/ARS plasmid (pRS413-npgA), successful gene introduction would result in heterologous rubrofusarin production. This tricyclic polyketide is visible as a distinct orange-brown pigment. Following transformation, six transformants formed colonies. These were evaluated for production by plating on an induction agar plate with 100 μM Cu^{2+}, and yellow pigmentation indicated the successful activity of the metabolic pathway as compared to a classically selected pathway strain and a negative control strain (Fig. 5B). Further, to verify introduction of all three plasmids, specific PCRs were performed on extracted DNA from three randomly chosen transformants and the resulting bands confirmed presence of all plasmids (example Fig. 5C). Accumulation of the two pathway intermediates result in yellow-toned though distinguishable pigmentation, for which reason PCR validation was particularly important as final proof of successful gene introduction.

Since five simultaneously propagated centromeric plasmids can cause considerate cytotoxicity (Futcher and Carbon, 1986), this was further evaluated in a strain with four empty CEN/ARS plasmids. Without selection, this strain did show loss of plasmids in long-term cultures, but normal cell morphology and exponential growth rate (supplementary material). With selection, the stability of the four CEN/ARS plasmids carrying the rubrofusarin pathway was further tested in ten randomly picked colonies following a 30-generation liquid cultivation. PCR on unique plasmids was confirmative for all four plasmids in all ten colonies, whereas maintained pathway function was indicated through visual inspection of cell pigmentation (supplementary material).

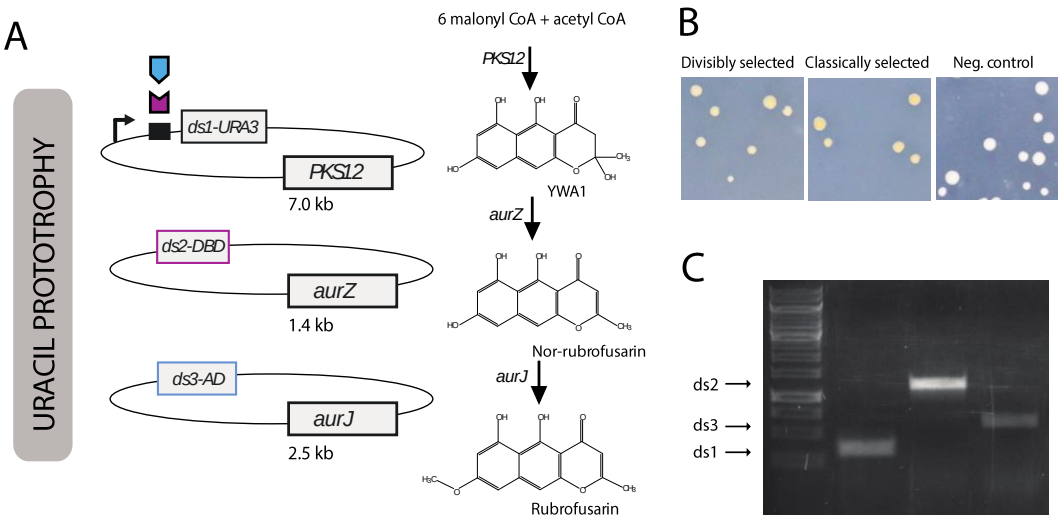

Figure 5 Introduction of a three-gene metabolic pathway in *S. cerevisiae* on three individual plasmids through a single transformation and selectable trait. A) The biosynthetic genes for the *F. graminearum* rubrofusarin polyketide pathway were introduced on each of the three divisible selection vectors to catalyze the indicated reactions from acyl-CoA precursors to rubrofusarin. B) Platings of *S. cerevisiae* strains with the rubrofusarin pathway maintained through i) one divisible selection phenotype (Ura^+) ii) three classical selection phenotypes (Ura^+, Leu^+, Trp^+) and iii) a reference CEN.PK2-1C strain with empty plasmids. Plated on respectively SC –ura-his, SC-ura-his-leu-trp and SC -ura-his-leu-trp agar plates, all with 100 µM Cu^{2+} for pathway induction. C) Verification of presence of all three divisible selection plasmids after transformation with rubrofusarin pathway by specific PCRs on genomic DNA. PCRs loaded for each of the three divisible selection plasmids yielding the expected and indicated band sizes. GeneRuler 1 kb loaded as size ladder.

4 Discussion

Divisible selection is a new modular concept for selection of several physically independent DNA fragments using the same selective trait. This yields increased

strain construction freedom since multiple, independent DNA can be introduced utilizing only one selectable phenotype. Such divisible selection is especially relevant in strains with limited selectable traits. Due to the modularity of the concept, flexibility is added to the strain engineering process in several aspects. Specifically, we have shown that the particular phenotype can be modularly swapped by only exchanging the selection gene ORF of the system (*ds1*). Further, while all three modules are essential, they need not all carry a pathway gene. For example, it is possible to utilize only the *ds2* and *ds3* modules (containing the hybrid DBD and AD parts) for production genes and co-transform *ds1* as phenotypic decider to control selection phenotype conveniently without sub-cloning. Such flexibility could become important in saving time needed to make pathway genes compatible with new host strains e.g. when testing performance in genetics-limited industrial strains or combining two individually tested segments of a long metabolic pathway.

ds1 is the critical component to the performance of the system by carrying the full selection gene ORF. As shown, there is a high probability that a single picked clone will be a correct transformant, since false-positive transformants on average occurred at 4-6 percent of the positively transformed clones. This low percentage could have been overestimated slightly from the assay method of omitting one of the split hybrid TF modules: due to the high co-transformation efficiency in *S. cerevisiae*, it is possible that a fraction of these false-positive cells would also take up additional plasmids if available. Whereas the plasmid copy number is maintained stably through cell divisions by the CEN sequence (Clarke and Carbon, 1985), it is possible that the few false-positive transformants may have arisen from uptake of multiple *ds1* copies

during transformation. In contrast the copy number variation of 2-micron plasmids led to an increased number of false positives. This episomal plasmid fluctuates considerably in copy number due to its propagation method (Mead et al., 1986) and thus also appears less ideal for metabolic engineering in some regards (Jensen et al., 2013). With targeted chromosomal integration, the introduction of a single copy of each *ds* module likely resulted in the high selection stringency observed and similar to that observed with the CEN/ARS plasmids.

The proof of principle by introduction of a three-gene fungal polyketide pathway demonstrates the potential of divisible selection to introduce metabolic pathways under a single selection phenotype. The polyketide pathway was chosen as a relatively large extreme in terms of size (total of 11 kb) and the size mainly resulted from the 7 kb polyketide synthase cassette. Since divisible selection depends on co-transformation of all DNA units in a single step, good transformation efficiency is important. For more extreme sizes, it is therefore possible that the use of simple lithium-acetate transformation methods will prove limiting, calling for methods suited for large DNA fragments. Ideally, future systems should improve the transformation colony formation rate compared to classical selection genes, e.g. through manipulation of the output promoter to increase responsiveness to the TF.

An alternative approach to transcriptional division of selection genes would be to split the individual ORFs encoding the selection genes to form individually unfunctional heteromultimers as demonstrated in *E. coli* (Schmidt et al., 2012). At a cost of modularity however, such approaches require that the splitting points of each individual selection gene are developed. For use in pathway construction, detailed

characterization of false-positive ratios in transformation is also important to understand the risks for picking these. Still, split TF-based divisible selection could be combined with such approaches to yield more co-dependent selection units. Alternative to transcriptional splits, longer nutrient biosynthesis pathways could be utilized for co-dependent selection, e.g. by building up selection upon several uracil biosynthesis genes. The disadvantage of such approaches, however, would be less flexibility and the requirement for pre-adapting strains, which limits strain compatibility with existing selection procedures.

By depending on split TF reconstitution, it may further be possible to break down the different modules to allow additional independent DNA fragments to be selected under the same phenotype. For CEN/ARS-based propagation however, the limit for stable maintenance may lie below five plasmids per strain (Futcher and Carbon, 1986). This thus favors chromosomal integration for multiple gene introductions. Similarly constraining could be the co-transformation efficiency or number of possible TF heteromers. The split of phage T7 RNA polymerase into multiple units (Segall‐Shapiro et al., 2014) is e.g. of relevance for future *E. coli* systems. High specificity of such DNA and protein interactions should enable orthogonal use of multiple co-selecting modules, i.e. through further co-dependent hybrid TFs, which independently control different selection gene ORFs. Such orthogonally divided selections could possibly extend the number of gene introductions possible before selection gene recycling.

New modular selection approaches that rethink selection are likely to significantly aid the speed of multi-gene evaluation. Introduction strategies such as the homologous recombination-guided DNA assembler method (Shao et al., 2009) provides advantages in terms of speed, but reliance on homologous recombination requires absence of interfering recombination-prone sequences. Recent gene editing methods relying on clustered regularly interspaced short palindromic repeats (CRISPRs) are also likely to help speed up cell factory development (DiCarlo et al., 2013; Jakočiūnas et al., 2015), especially if off-target effects or intellectual property concerns (Sherkow, 2015) are not significant or relevant. Selection schemes further have metabolic engineering applications within synthetic screens and other high-throughput assays (Dietrich et al., 2010).

Finally, divisible selection acts as a multi-component AND gate in the sense that all modules are required to transmit the signal (of DNA uptake in this case). Since selection is a powerful method for enhancing multi-component screening, it is possible that the general split hybrid TF network could take other applications, e.g. adapted to other synthetic signal-processing schemes requiring AND logics.

In conclusion, a divisible selection concept based on split hybrid TFs was developed to extend the single utility of selection genes to three. Such systems could be particularly well suited for providing combinatorial flexibility when operating with centromeric plasmids. As shown, the concept could also be used for utilizing the benefits of chromosomal gene integration.

5 Competing interests

The authors declare no competing interests.

6 Author contributions

PR and MOAS conceived the study. NMP and PR performed the experiments. PR, NMP and MOAS wrote the manuscript.

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Supplementary material for:

Flexible metabolic pathway construction using modular and divisible selection gene regulators

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Table of Contents

S1 Construction of plasmids

Plasmids were all assembled from individual PCR fragments. Table S1 lists the PCR fragment composition (by primers and DNA template) leading to the indicated plasmid. Further detail on PCR templates is given in Table S2, while the respective oligonucleotide sequences are specified in Table S3.

Table S1 PCR fragments used to assemble the listed plasmids using the indicated primer pairs and DNA template. Sequences of linear DNA fragments used as template are given in text below, and overview of template plasmids is given in Table S2. All plasmids were assembled through uracil excision cloning with the exception of pDS2-aurZ.

Plasmid ID	Primer pair	Template
pDS1 U	P312/P313	pRS416
	P273/P258	pUG72
	P305/P56	*S. cerevisiae* gDNA
	P55/P302	*SPO13* hybrid DNA fragment
	P255/ID399	pUG72
pDS1 H	P285/P55	pDS1 U
	P205/P284	*S. cerevisiae* gDNA
pDS2	P313/P312	pRS416
	P261/P273	pUG75
	P192B/P192	*E. coli* gDNA
	P193/P283	pEXP32
	P260/ID399	pUG75
pDS3	P312/P313	pRS416
	P261/P273	pUG75
	P42/P112	VP16 AD DNA fragment
	P202/P259	pEXP22
	P260/ID399	pUG75
pS1.2	P55/P267	pDS1 U
	P252/P273	pUG72
	ID399/P255	pUG72

	P271/P272	pRS416
pS1.3	P273/P302	pDS1 U
	P255/P272	pS1.2
pDS1U-PKS12	P273/ID399	pDS1 U
	P312/P313	pRS416-PKS12
pDS2-aurZ Gibson assembly	P273/ID399	pDS2
	SmaI-digested plasmid	pRS414-aurZ
pDS3-aurJ	P273/ID399	pDS3
	P312/P313	pRS415-aurJ
pDS1Um	P116/P119	pDS1U
	P117/P118	pESC-HIS
pDS2m	P116/P119	pDS2
	P117/P118	pESC-HIS
pDS3m	P116/P119	pDS3
	P117/P118	pESC-HIS
pDS1U-X2	P468/ID399	pDS1U
	P467/P469	pCfB393
pDS2-X3	P468/ID399	pDS2
	P467/P469	pCfB394
pDS3-X4	P468/ID399	pDS3
	P467/P469	pCfB395

Table S2 Plasmids used as PCR template for plasmid construction (listed in Table S1) and alternative ds1 plasmids evaluated in S2.

Plasmid	**Features (not complete)**	**Reference**
pDS1.2U	pUC origin, f1, ARS/CEN, *ampR*, 8op$_{lexA}$-p$_{SPO13}$-*URA3*	This study
pDS1.3U	pUC origin, f1, ARS/CEN, *ampR*, 4op$_{lexA}$-p$_{SPO13}$-*KlURA3*	This study
pUG72	pUC origin, loxP-p$_{KlURA3}$-*KlURA3*-t$_{KlURA3}$-loxP, *ampR*	(Hegemann and Heick, 2011)
pUG75	pUC origin, loxP-p$_{AgTEF1}$-*hphMX*-t$_{AgTEF1}$-loxP, *ampR*	(Hegemann and Heick, 2011)
pEXP22	*RalGDS-GAL4AD*, pUC origin, f1, ARS/CEN, *TRP1*, *ampR*	Life Technologies - Proquest
pEXP32	*Krev1-GAL4DBD*, pUC origin, f1, ARS/CEN, *LEU2*, *ampR*	Life Technologies - Proquest
pRS413	pUC origin, f1, ARS/CEN, *HIS3*, *ampR*	(Sikorski and Hieter, 1989)
pRS414	pUC origin, f1, ARS/CEN, *TRP1*, *ampR*	(Sikorski and Hieter, 1989)
pRS415	pUC origin, f1, ARS/CEN, *LEU2*, *ampR*	(Sikorski and Hieter, 1989)
pRS416	pUC origin, f1, ARS/CEN, *URA3*, *ampR*	(Sikorski and Hieter, 1989)

pRS416-PKS12	p_{CUP1}-*PKS12*-$t_{ADH1,}$ pUC origin, f1, ARS/CEN, *URA3*, *ampR*	(Rugbjerg et al., 2013)
pRS414-aurZ	p_{TEF1}-*aurZ*-$t_{ENO2,}$ pUC origin, f1, ARS/CEN, *TRP1*, *ampR*	(Rugbjerg et al., 2013)
pRS415-aurJ	p_{GPD1}-*aurJ*-$t_{CYC1,}$ pUC origin, f1, ARS/CEN, *LEU2*, *ampR*	(Rugbjerg et al., 2013)
pESC-HIS	2-micron, *HIS3*, *ampR*	Agilent Technologies
pCfB393	X-2 targeting flanks, pTEF-CFP-tADH1, *ampR*	(Jensen et al., 2013)
pCfB394	X-3 targeting flanks, pTEF-RFP-tADH1, *ampR*	(Jensen et al., 2013)
pCfB395	X-4 targeting flanks, pTEF-YFP-tADH1, *ampR*	(Jensen et al., 2013)

Nucleotide sequence of SPO13 hybrid DNA fragment:

>SPO13 hybrid DNA fragment

TTCCGACCTGCAGTACTGTATGTACATACAGTACTGTATGTACATACAGTACTGCAGGCATGCAAGCT

CTAGAGTCTCCGTTTAGCTAGTTAGTACCTTTGCACGGAAATGTATTAATTAGGAGTATATTGAGAAA

TAGCCGCCGACAAAAAGGAAGTCTCATAAAAGTGTCTAACAGACAATTAGCGCAATAAGAAGAAAGAA

AACGGATTGAAGTTGAGTCGAGAATAATT

Nucleotide sequence encoding VP16 AD, synthesized by Life Technologies GeneArt:

>VP16 AD DNA fragment

TCGACGGCCCCCCCGACCGATGTCAGCCTGGGGGACGAGCTCCACTTAGACGGCGAGGACGTGGCGAT

GGCGCATGCCGACGCGCTAGACGATTTCGATCTGGACATGTTGGGGGACGGGGATTCCCCGGGGCCGG

GATTTACCCCCCACGACTCCGCCCCCTACGGCGCTCTGGATATGGCCGACTTCGAGTTTGAGCAGATG

TTTACCGATGCCCTTGGAATTGACGAGTACGGTGGG

Table S3 Sequence of oligonucleotides used in the study

Primer ID	Oligonucleotide sequence (5'-)
P35	GACAGCTTCCTGATCGGAAGG
P42	AACATAUGCCCAAGAAGAAGCGG
P55	AATTATTCUCGACTCAACTTCAATC
P56	AGAATAATUATGTCGAAAGCTACATATAAGGAA
P111	AGCGGAAGGUCAAGCTTTCGACGGCCCCC
P112	ACCCACCGUACTCGTCAATTCC
P116	AGGTGGCACUTTTCGGG
P117	AGTGCCACCUGAACGAAG
P118	ATGGTTTCUTAGATGATCCAATATC
P119	AGAAACCAUTATTATCATGACATTAAC
P191	AGCGGAAGGUCTCGAGCATGAAAGCGTTAACGGCCAG
P192	AGCCAGUCGCCGTTGCG

P192B	AACATAUGCCCAAGAAGAAGCGGAAGGTCTCGAGCATGAAAGCGTTAACGGCCAG
P193	ACTGGCUGAGGTCGAATCAAACAAGTTTG
P202	ATTGTCGAAUCAAACAAGTTTGTA
P205	AGAATAATUATGACAGAGCAGAAAGCC
P230	ACCCTTTCUCCGCTTCTTTAGGATGAAGTCATAGTTGGC
P252	ATTATGUCCACAAAATCATATACCA
P255	ACGAGCUTTCGAGAACCC
P258	AGCTTAUACAGGAAACTTAATAGAACAAATC
P259	ACCGCTUCTTTAGGATG
P260	AAGCGGUAACTCGAGGACAATAAAAAGA
P261	ATATGTUCTTGTTTATGTTCGGATGTG
P267	AGCTCGUTCCGACCTGCAGTACTG
P271	ACCCAAUGCATCAGGAAATTGTAAACG
P272	AAGCTGUGGTATGGTGCA
P273	ACAGCTUTCGACAACCCTTAATATAACTTC
P283	ACCGCTUCTAGAGCAGCAGACATGATTTC
P284	ATTACAUAAGAACACCTTTGGTG
P285	ATGTAAUTATACAGGAAACTTAATAGAACAAATC
P302	AGCTCGUCTGTATATATATACAGATCTGATATATATACAGCGTTCCGACCTGCAGTACTG
P377	ACTGAGAUACCTACAGCGTGAGCTAT
ID399	ATTGGGUGCATAGGCCACTAGTGGATCTG
P467	ACGCGAUCTTCGAGCGTCCCAAAACC
P468	ATCGCGUCATAACAGCTTTCGACAACCC
P469	ACCCAAUTCGCCCTATAGTGAGTCG

S2 Evaluation of alternative *ds1* modules

To investigate a possible advantage of utilizing a heterologous nucleotide sequence of *URA3*, we designed a mutant *ds1* module with a heterologous selection gene ORF the *K. lactis URA3* (ds1.3-KlURA3), which has limited identity to potential host-inactivated *URA3* genes in *S. cerevisiae* laboratory strains.

At prolonged incubation times following transformation (30 °C for 6 days), this plasmid alone however permitted formation of small false-positive colonies (Fig S1). We therefore tested the *S. cerevisiae URA3* in a new construct to limit these false-positive colonies and possibly improve true positives by simultaneously introducing extra high-affinity LexA binding sites. Together with *ds2* and *ds3*, the *ds1.2* module (pDS1.2U) produced an average of 126 transformants following three days incubation and alone 2 false-positive transformants on average, while the second *ds1.3* module (pDS1.3U) with two additional LexA binding sites and *S. cerevisiae URA3* generated the same average of false- and true-positive colonies (Fig. S1). Interestingly however, the use of *URA3* from *S. cerevisiae* did not lead to elevated false-positive colony formation at prolonged incubation times. This indicated a possible lower background expression or activity of this URA3 enzyme and also that the few false-positive colonies did not result from recombination at the inactivated *ura3* locus of the transformed CEN.PK2-1C strain.

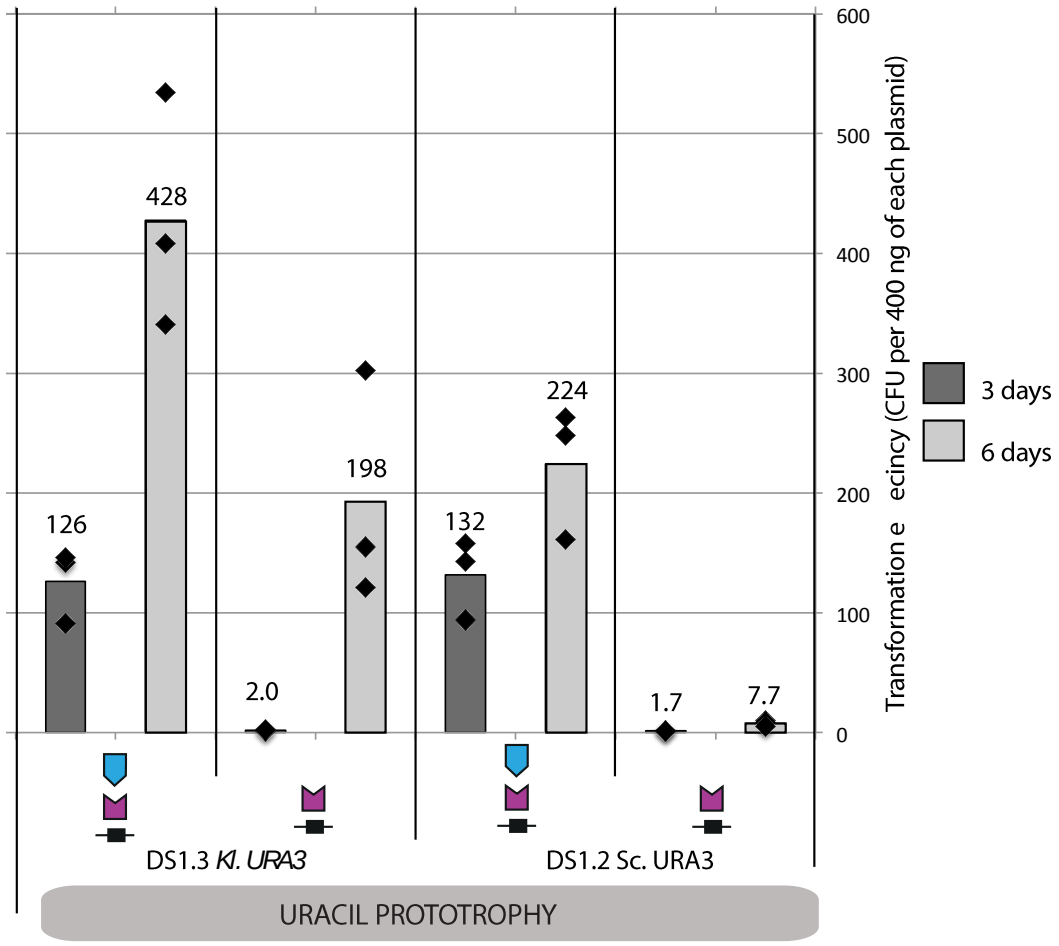


Figure S1. Characterization of *ds1* modules at prolonged incubation. Transformation efficiencies (average CFU per 400 ng of each plasmid) in *S. cerevisiae* CEN.PK2-1C transformed with incomplete or complete divisible selection modules (*ds1, ds2 and ds3*) under selection on SC –ura. CFUs counted following three or six days at 30 °C. Black points show values from individual replicate transformations (n = 3).

S3 Stability of four CEN6-propagated plasmids

The viability of strains propagating four CEN6/ARS plasmids in a *S. cerevisiae* CEN.PK2-1C strain was assayed in comparison to strains transformed with respectively zero and one CEN6/ARS plasmid (Table S4).
To test if a cost of propagating the number of plasmids was conferred to the strains, their growth rates were first measured. In order to avoid potential influences of the different prototrophic genes on growth rate (Pronk, 2002), which would not be a measure of CEN6/ARS plasmid toxicity, the evaluation of the growth rates was performed in non-selective YPD medium, which would also allow loss of plasmids. Empty plasmids were used to exclude fitness costs associated with expressing various numbers of pathway enzymes. Pre-cultures of the strains grown in selective SC medium were used to inoculate 180 µL microtiter cultures in YPD medium, at 30 °C, 300 rpm horizontal shaking (New Brunswick Innova 44R). Their growth rates were calculated by exponential regression of OD600 values versus time obtained in the same range of the exponential growth phase and including at least seven data points. The resulting similar growth rates in non-selective YPD medium (Table S4) indicated that four different CEN6/ARS plasmids did not constitute a significant growth disadvantage in the CEN.PK2-1C strain without selection for plasmid maintenance. Loss of plasmids over time would however occur (Clarke and Carbon, 1985).

Table S4 Growth rate of *S. cerevisiae* CEN.PK2-1C harboring respectively 0, 1 and 4 different CEN6/ARS plasmids, in exponential phase, cultured in YPD at 30 °C , 300 rpm horizontal sharking (n = 3).

***S. cerevisiae* strain**	**Number of different CEN6/ARS plasmids**	**Growth rate (hr^{-1}) +/- standard deviation**
CEN.PK2-1C	0	0.27 +/- 0.0032
CEN.PK2-1C + pRS413	1	0.26 +/- 0.0080
CEN.PK2-1C + pRS413 + pDS1U, pDS2, pDS3	4	0.27 +/- 0.0061
CEN.PK2-1C + pRS413 +	4	0.26 +/- 0.0095

pRS414 + pRS415+ pRS416		

The degree of plasmid loss was subsequently indicated through plating of serially diluted spots of a four CEN6/ARS plasmid strain on various, selective SC media (SC, SC -histidine, SC –uracil -histidine) from non-selective YPD cultures grown at 30 °C**,** for respectively 20 and 30 generations through passing of 15 mL cultures to fresh medium (Figure S2). Whereas maintenance of only one CEN6/ARS plasmid appeared stable without selection (plated on SC – histidine), a significant number of cells had lost at least one of the four plasmids, as seen when selecting for all simultaneously (SC – histidine, -uracil) (Figure S2). This implies that selection for four plasmids constitutes a negative factor on the growth rate of strains harboring four CEN6/ARS plasmids (overexpression of their respective selection genes could be another factor). In expanding divisible selection concepts beyond three centromeric plasmids, these effects would be important to characterize in depth.

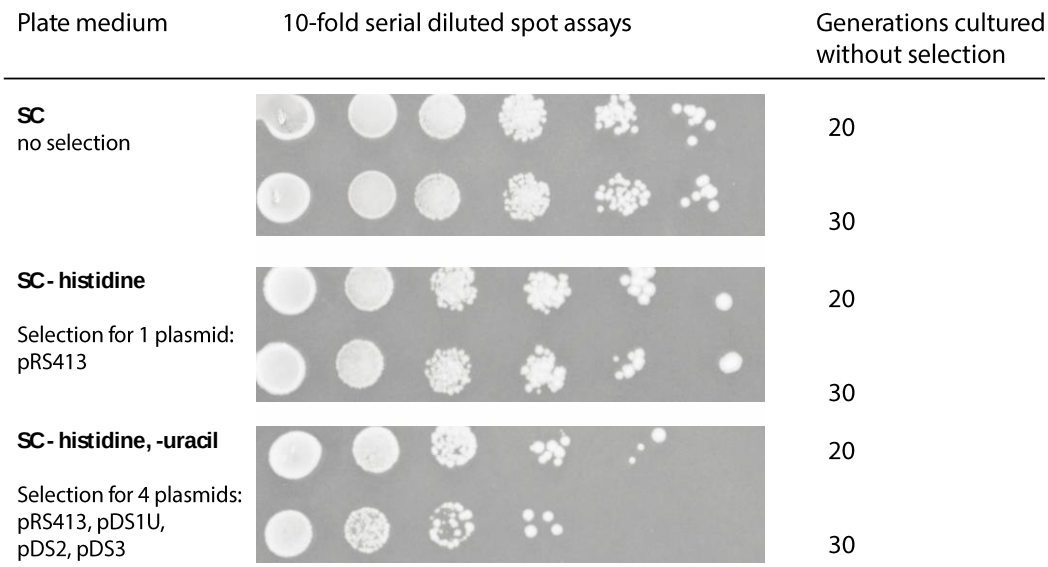


Figure S2 Loss of plasmids following long-term incubation in non-selective medium. 10-fold serial dilution spot assays of *S. cerevisiae* CEN.PK2-1C transformed with four plasmids, on the indicated plates selecting for the number of CEN6/ARS plasmids shown to evaluate the number of colony-forming cells following incubation for the indicated number of generations in non-selective YPD medium. Plates incubated at 30 °C for 4 days.

The cell morphology in cultures grown in selective SC medium at 30 °C was finally inspected by microscopy to assess the health of the four-plasmid cells. Visual comparison showed no clear morphological differences nor long filament-like cell structures (Figure S3) as observed in strains with five CEN3/ARS plasmids in the *S. cerevisiae* BF307-10 strain grown in selective YNB medium (Futcher and Carbon, 1986). The size of the cells containing four CEN6/ARS plasmids could however appear slightly larger than of those containing zero.

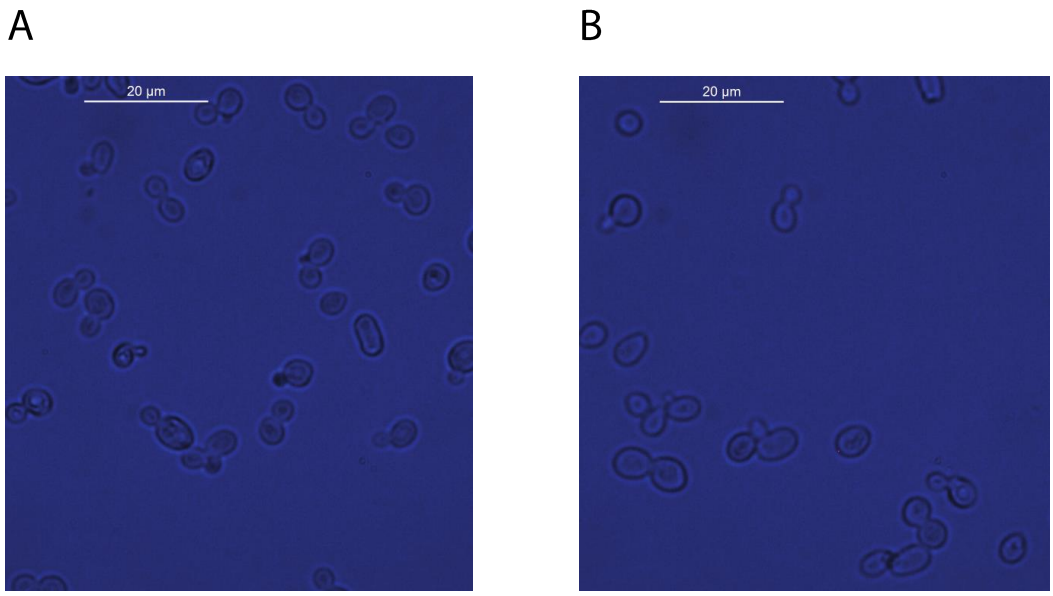


Figure S3 Cell morphology. Exponentially growing *S. cerevisiae* cells in selective SC medium transformed with respectively A) zero CEN6/ARS plasmids and B) four different CEN6/ARS plasmids.

To test the ability to maintain production with a biosynthetic pathway, cultures of divisibly selected rubrofusarin strains (ds-rub) were grown in selective SC medium (-uracil, -histidine) for 30 generations and plated on selective plates. The stability of each of the four CEN6/ARS plasmids was then tested in ten randomly picked colonies through PCR of unique elements (Table S5). Specific PCR products from all four plasmids could be detected in all ten colonies. The corresponding ability to maintain production was also indicated by their pigmentation through re-streak of the same ten colonies to plates with induction of the pathway (Figure S4). The yellow nuance of the rubrofusarin pathway precursors (YWA1 and nor-rubrofusarin) however means that the PCR-based evaluation is important to verify maintenance of all plasmids.

Table S5 PCR confirmation of plasmid maintenance in ten colonies of rubrofusarin-pathway *S. cerevisiae* following 30-generation liquid cultivation. For each plasmid, the oligonucleotide pairs used in PCR are shown with their results.

Plasmid to be verified	Oligonucleotides used	Colonies with plasmid confirmed
pRS413-npgA	P35 + P377	10/10

pDS1U-PKS12	P55 + ID399	10/10
pDS2-aurZ	P191 + P283	10/10
pDS3-aurJ	P111 + P230	10/10

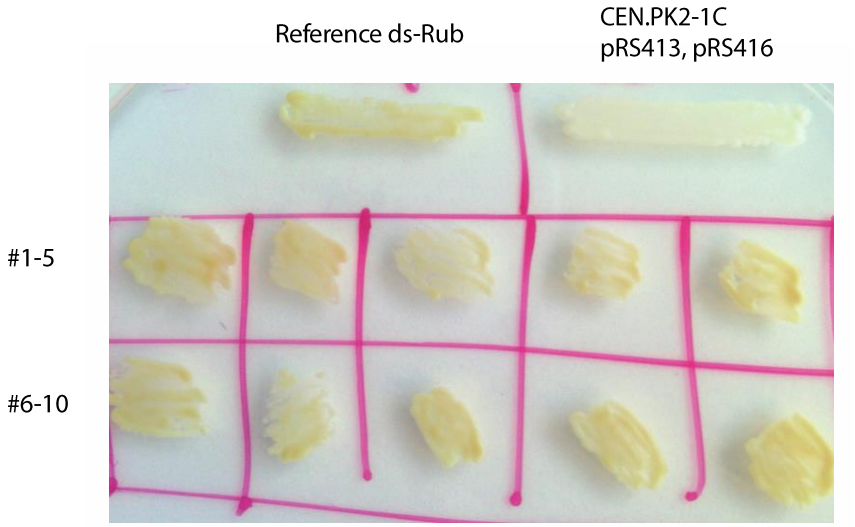
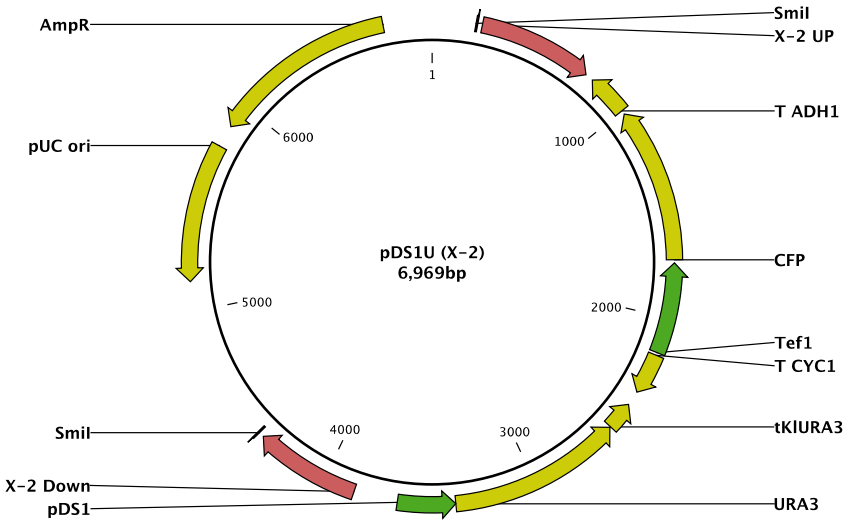
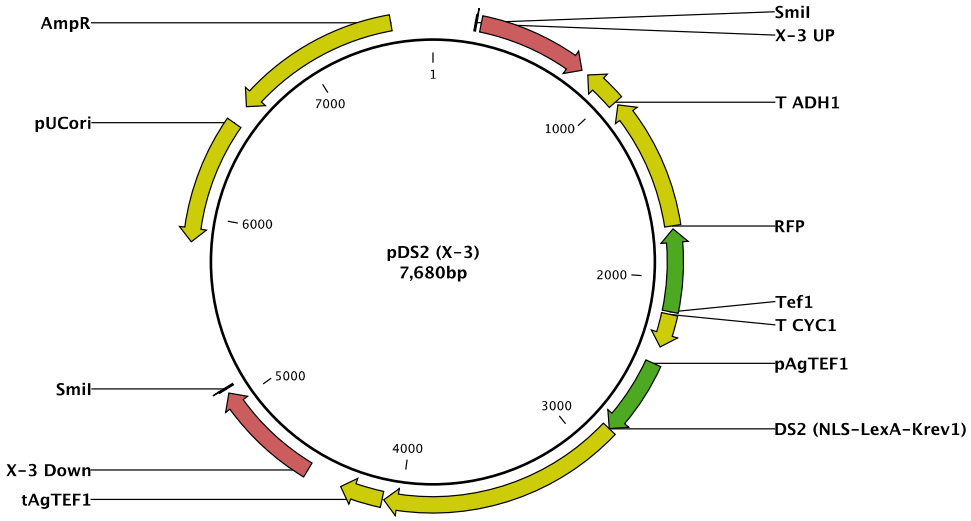
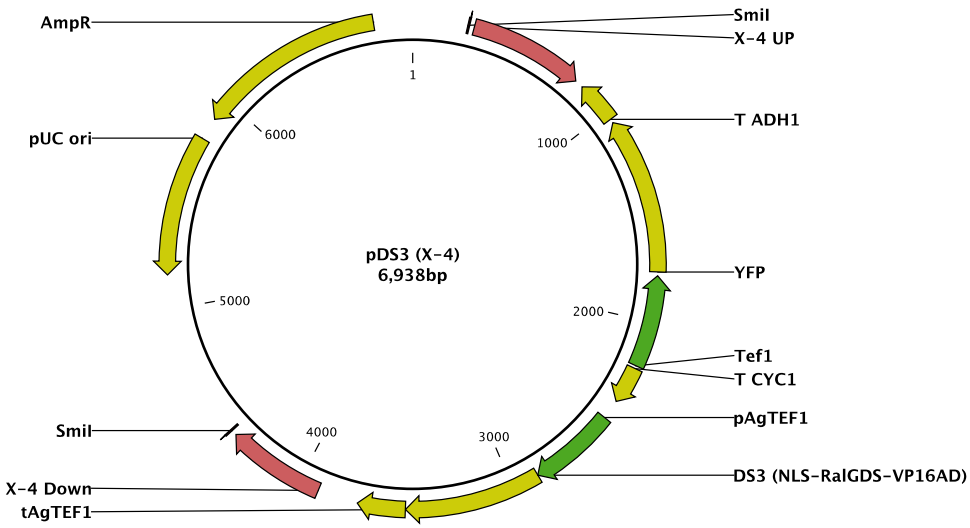


Figure S4 Confirmation of pathway product accumulation of individual colonies picked following long-term culture. Individual colonies streak to SC –uracil, -histidine plates with 100 µM Cu^{2+}, incubated for 4 days at 30 °C. Photograph brightness was increased 20 percent.

S4 Plasmid maps







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